Research Article

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Factors Influencing the Successful Isolation and Expansion of Aging Human Mesenchymal Stem Cells

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Abstract: Most studies highlight mesenchymal stem cells (MSCs) extracted primarily from bone marrow (BM), very few report the use of peripheral blood (PB), often due to the associated low seeding density and difficulties with extraction techniques. As ageing populations are becoming more predominant globally, together with escalating demands for MSC transplantation and tissue regeneration, obtaining quality MSCs suitable for induced differentiation and biological therapies becomes increasingly important. In this study, BM and PB were obtained from elderly patients and extracted MSCs grown *in vitro* to determine their successful isolation and expansion. Patients' socio-demographic background and other medical information were obtained from medical records. Successful and failed cultures were correlated with key demographic and medical parameters. A total of 112 samples (BM or PB) were used for this study. Of these, 50 samples (44.6%) were successfully cultured according to standardised criteria with no signs of contamination. Our comparative analyses demonstrated no statistical correlation between successful MSC cultures and any of the six demographic or medical parameters examined, including sample quantity, age, sex, race, habits and underlying comorbidities of sample donors. In conclusion, the present study demonstrates that typical demographics and comorbidities do not influence successful MSC isolation and expansion in culture.

Keywords: Mesenchymal stem cells; Ageing, Bone marrow; Peripheral blood; Demographic factors; Comorbidities; Contamination; Mononuclear cells; Density gradient centrifugation

1 Introduction

Mesenchymal stem cells (MSCs) have been studied in depth owing to their self-renewal capacity and multi-potentiality with possible application in the treatment of a variety of medical conditions through advances in tissue engineering and regenerative medicine in recent years [1]. MSCs are found to reside primarily in the stem cell niche of bone marrow (BM), but may also exist in other sites such as adipose tissue, umbilical cord blood and even peripheral blood (PB) [2, 3].

Interestingly, studies have shown conflicting data on the isolation of MSCs from peripheral blood. Zvaifler *et al*. have indicated that mesenchymal progenitors are present, albeit at low density, in normal adult peripheral blood [3]. However, Wexler *et al*. failed to confirm the existence of MSCs in PB [4]. Recently, some studies have documented that peripheral blood-derived mesenchymal stem cells (PB-MSCs) possess biological characteristics which are similar to that of bone marrow-derived mesenchymal stem cells (BM-MSCs) [5, 6]. Thus, advancements in techniques for MSC extraction from peripheral blood - a clinically accessible potential source of stem cells involving a minimally invasive procedure, compared to bone marrow, still need further investigation and development.

When stimulated by specific signals, MSCs are released from their BM niches into the circulation and may be recruited by target tissues, where they undergo *in situ* differentiation and contribute to tissue regeneration and homeostasis. These stem cells possess high proliferation

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potential, can be manipulated easily permitting directed differentiation prior to implantation for tissue repair purposes. In many reported studies [7, 8], the requirement of high cell seeding densities has proven to be the basis for successful biological therapies, hence it has become important that expansion either through *in vitro* or *ex vivo* be achieved at all cost.

However, successful cell expansion has not always been the norm as described in several studies, even by established laboratories [9, 10]. Specificity of factors that contribute to successful cell expansion *in vitro* is largely speculative, although certain factors have been deemed more likely to cause culture growth during the replication stages. This has led to the view that perhaps patient factors such as age and tissue sources are a major concern and thus only MSCs obtained only from certain patients are suitable for therapy [11, 12]. Health conditions such as diabetes have also been implicated but never substantiated in any known study [13]. Thus, this study was undertaken to determine demographic, lifestyle and intrinsic factors of patients that may contribute to the success and failure rates of MSC cultures, therefore possibly elucidating stricter selection criteria for MSC-based therapies in future.

2 Materials and Methods

Experiments conducted using human mesenchymal stem cells harvested from bone marrow and peripheral blood of patients were approved by the Medical Ethics Committee, University of Malaya (Reference number: 369.19). After obtaining informed consent from all individuals included in this study, a total of 112 samples, BM or PB, were collected. Bone marrow and peripheral blood specimens were collected from different individuals. Bone marrow aspirates were drawn and collected by orthopaedic surgeons (co-investigators) from medullary canals of the femur or iliac crest during hip or knee surgeries at the University of Malaya Medical Centre (UMMC). Peripheral blood samples were obtained through routine venepuncture (via upper limb veins) and collected into 10 ml vacuum blood collection tubes containing 18 mg K2EDTA anticoagulant (BD Vacutainer, BD-Plymouth, UK).

Records of all tissue samples sent for cell culture were analyzed as part of a cross-sectional study at UMMC. MSCs were isolated, characterized and identified using previously established protocols [14]. Patient data including socio-demographic background information and other relevant medical history details were obtained from the Patient Information Division, UMMC. Medical records were reviewed and analysed with regard to demographic data such as age, sex and ethnicity. Other factors such as the patient's medical condition (with/without co-morbidities), tissue source, quantity of sample as well as tobacco use status were also recorded and analysed. Variables related to patient tissue source were compared for resultant successful and non-successful cell cultures, using a Pearson chi-square test for discrete data, with P value < 0.05 as being significant. Other correlation studies using Spearman and/or linear or multiple regression analyses were tested where possible. Data was analyzed using SPSS, Version 14 for Windows.

Informed consent: Informed consent has been obtained from all individuals included in this study.

Ethical approval: The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by Medical Ethics Committee, University of Malaya.

3 Results

Depending on the bone origin, BM samples may contain lipid droplets (Figure 1A). However, regardless of lipid presence, layers containing mononuclear cells were still observed following the density gradient centrifugation method (Figure 1B). MSCs were successfully isolated from human BM and PB and adherent cells observed in suspension under microscopy (Figure 2). Data for the characterization and identification of BM and PB-derived MSCs have been reported in our previous study following the criteria described in The International Society for Cellular Therapy position statement [3, 15]. In brief, the isolated BM-MSCs met the three inclusion criteria: (i) adherence to plastic when maintained in culture; (ii) positive expression of several types of antigens such as CD29, CD105, CD166 but negative to others such as CD34 and CD45; (iii) the ability to differentiate along multi-lineages including into osteoblasts, adipocytes and chondrocytes under *in vitro* inductive conditions.

A summary of the demographic data and the success rates of MSC cultures are presented in Table 1. Of the 112 samples, there were 50 (44.6%) successful cultures of MSCs isolated from either BM or PB specimens. The unsuccessful cultures due to bacterial contamination are shown in Figure 3. In this cross-sectional study, there were no significant differences in the success of cultures when

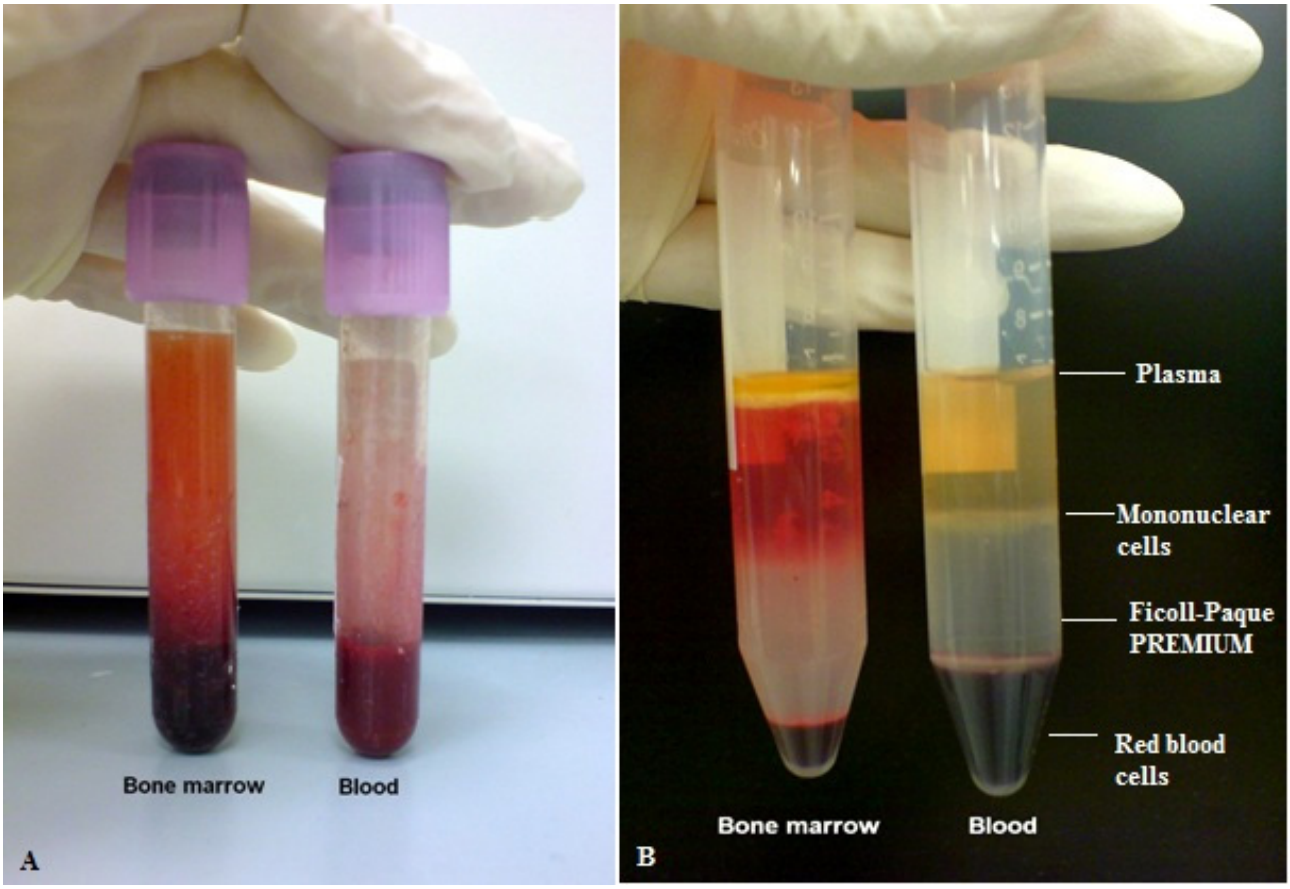

Figure 1: Bone marrow and peripheral blood samples were collected and mononuclear cells extracted and separated using Ficoll-Paque PREMIUM via centrifugation. (A): Typical original bone marrow and peripheral blood samples collected from patients. (B): Comparison of original bone marrow and peripheral blood samples after mononuclear cell extraction and separation using density gradient centrifugation method.

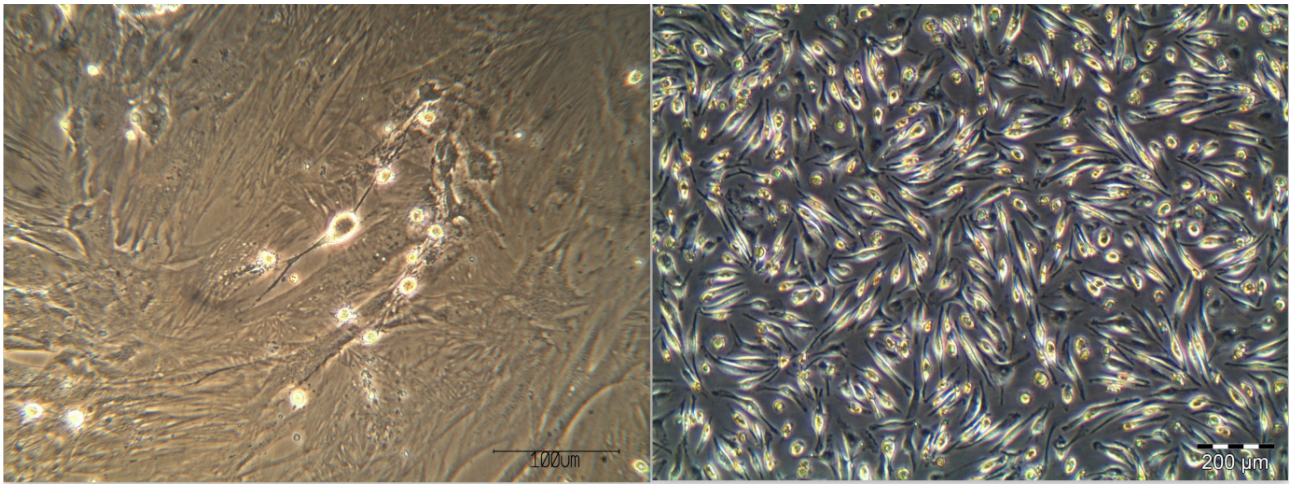

Figure 2: Morphology observation of (A) BM-MSCs with prominent nuclei and spindly cytoplasmic processes and (B) PB-MSCs with fibroblast like appearance.

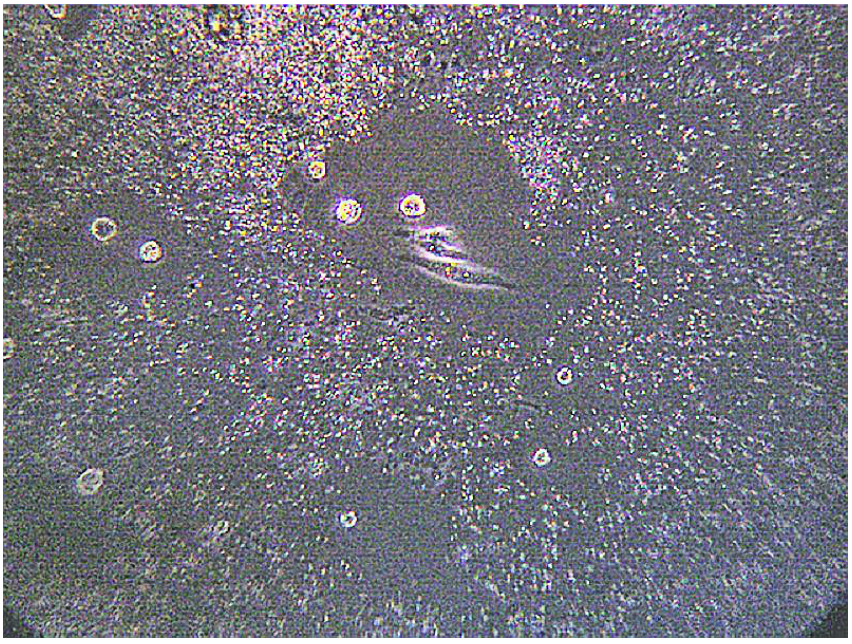

Figure 3: Morphology observation of contaminated PB-MSCs surrounded by bacteria.

Table 1: Summary of key variables that determine the success rates of isolation and expansion of MSC cultures

Variables	Successful cultures (n=50)	Non-successful cultures (n=62)	P-value
Age (Years)	61.4±11.9	58.5±14.2	0.258
Sex (M:F)	19:31	20:42	0.526
Race (M:C:I:O)	15:25:6:4	14:34:10:4	0.769
Sample type (BM:PB)	18:32	28:34	0.327
Sample volume (<1:1-1.9:2-3ml)	5:7:38	6:18:38	0.158
Co-morbidities (None:Present)	40:10	45:17	0.361
Habits (None:S:A:SA)	43:4:1:2	53:5:2:2	0.977

Abbreviations: Sex (M – Male, F – Female), Race (M – Malay, C – Chinese, I – Indian, O – Others), Sample type (BM – Bone marrow, PB – Peripheral blood), Habits (S – Smoker, A – Alcohol consumer, SA – Smoker and Alcohol consumer)

correlated with sample source, age, sex or race (Malays, Chinese, Indians and other races). The majority of donors were mainly patients diagnosed with Osteoarthritis (OA) undergoing some form of hip or knee surgery.

Another variable assessed was the sample volume collected which ranged between 0.5 ml to 3 ml. Eleven samples (9.8%) were less than 1 ml in volume, 25 samples (22.3%) were between 1-1.9 ml and 76 samples (67.9%) were between 2-3 ml in volume. The volume of samples collected for all three groups (<1: 1-1.9: 2-3 ml) was not significantly different. When the sample volume obtained was less than 1 ml, 5 cultures were successful and 6 cultures were unsuccessful. When the sample volume obtained was between 1-1.9 ml, 7 cultures were successful and 18 were unsuccessful. With sample volume between 2-3 ml, 38 samples were successful whilst 38 samples were not. The data showed that sample volume did not determine the success of MSC cultures (p=0.16). Likewise, there were no statistically significant differences between the success rates of cultures from samples obtained from patients with co-morbidities and those without (P=0.36). Comparative analysis of patient habits such as smoking and alcohol consumption patterns showed no significant effect on the success rate of MSC cultures (P=0.977). The above results suggest that changes in demographic factors, disease condition and smoking/alcohol habits did not influence the success rate of MSC culture in this study. In this cross-sectional study, the result showed no statistical significant differences between the success rates of MSC cultures extracted from younger, middle-aged or older patients (P=0.26).

4 Discussion

Some studies have reported an age-related decrease in the proliferation and differentiation ability of MSCs [16]. In addition, some studies demonstrated a higher number of proliferative precursor cells could be observed in younger subjects [17-19]. On the other hand, other studies concluded a lack of age-related decreases in the proliferative and differentiation ability of MSCs [20]. The variation in the previous outcomes may be attributed to the inconsistency and insufficient power inherent in the methods used to detect the success or failure of cell cultures. The different outcomes related to the present study compared to those previously published may also be attributed to the fact that the tissue donors belonged to middle and older age groups, which may have skewed the data and possibly also inherently influenced the interpretation of this study results.

Gender did not prove to be a determinant factor for the success and failure rates of the isolation and expansion of MSCs. This was contrary to results reported by Muschler *et al* [21]. Although the distribution of samples obtained between males and females is relatively non-symmetrical, i.e. thirty-nine males vs. seventy-three females, an equal number of success and failure rates of cultures from male and female populations were observed. There was also no statistically significant differences between the success rates of MSC cultures obtained from Malays, Chinese, Indians or other races. This was true even when the data were stratified according to the different sexes (P=0.769). In the current study, it was also noted that sample volume did not significantly correlate with the likelihood of success in isolating MSCs (P=0.16). It was found that no specific volume was deemed optimal for the isolation and expansion of MSCs. In fact, larger volume aspirates may result in reduced MSCs that can be potentially obtained, which may also be viewed as unnecessarily wasting precious bone marrow [22]. Other factors that may have contributed to the failure of isolation or culture such as those reported by Michot and Gut [23], Casagrande and Michot [24], and Tseng *et al.* [25] include high alcohol intake shown to inflict damage to BM and PB mononuclear cells, and chronic smokers possessing reduced progenitor cells [26]; was not proven to be so. These also included other factors such as underlying co-morbidities.

Based on our findings of the isolation and expansion of MSCs, this would seem to suggest that the failure rates are unlikely to be due to the demographic background and co-morbidities. Rather, failed cultures were mostly due to sample contamination probably caused by improper aseptic techniques applied during peripheral blood specimen collection. The venepuncture skin site should be cleaned with povidone followed by an alcohol swab in a circular motion from the center of the area outwards. The caps of vacuum blood collection tubes should also be cleaned with povidone or alcohol. The blood should be collected from each patient directly into the vacuum blood collection tubes using a "butterfly" (Safety-Lok Blood Collection Set, 23G) or multi sample needle. Any contaminated blood specimens (either caused by microorganisms from exposed air or yeast contamination from the donor's skin) cannot be used for further cell culture study. In addition, the blood specimens have to be maintained at 4°C and mononuclear cell isolation has to be performed within 8 hours in order to maintain MSC viability.

In addition, bone marrow aspirates were drawn and collected from the medullary canals of the femur or iliac crest during bone grafting or fracture fixation procedures. The collected bone marrow samples may not always contain representative pure bone marrow as compared to bone marrow aspirates using a Jamshidi™ needle. The collected bone marrow samples may dilute with lipid rich fat, peripheral blood or mobilization of cells from the marrow, which would contribute to the low number of successfully cultured samples.

As the current study is limited by a relatively small sample size, we were unable to carry out multivariate analysis, which would have given more reliable statistical analysis. In addition, it needs to be noted that the average age of sample donors was 60 years, which if compared to a much younger age group i.e. 30 years and below, may have yielded significant differences in MSC growth. This is recognised as an unavoidable study limitation due to ethical reasons and unavailability of young donor samples at the time this study was conducted. An aspect worth mentioning in this article is that there may be scepticism that MSCs may not be isolated easily from PB, since circulating MSCs in PB have been shown to be in low concentrations. However, it needs to be clearly mentioned that such results have been achievable in our lab and as such we were able to demonstrate this feat in our previous publication which confirms such findings [14].

5 Conclusion

In conclusion, demographic variables, co-morbidity status and type or volume of tissue sample do not appear to be predicting factors that determine the rate of success in isolating and expanding MSCs. This result however, has to be demonstrated in a larger cohort study involving multiple time points in order to be more definitive.

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Conflict of interest: Authors state no conflict of interest.

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